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Gene, 88 (1990) 37–45
Elsevier

GENE 03451

Outer-membrane PhoE protein of *Escherichia coli* K-12 as an exposure vector: possibilities and limitations

(Recombinant DNA; hybrid protein; cell surface; protein engineering; biogenesis; vaccines; pore protein; plasmid vector)

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Received by J.-P. Lecocq: 7 September 1989
Revised: 3 November 1989
Accepted: 9 November 1989

SUMMARY

The phosphate-limitation-inducible outer-membrane protein (PhoE) of *Escherichia coli* K-12 can be used in an expression system as a carrier for foreign antigenic determinants, facilitating their transport to the bacterial cell surface. The system is very flexible, since insertions varying in length and nature can be made in different cell-surface-exposed regions of PhoE protein, without interfering with the assembly process into the outer membrane. Multiple insertions of an antigenic determinant can be made in the second and eighth exposed regions, resulting in a total insert length of up to 30 and 50 amino acid (aa) residues. Insertions can be made in two exposed regions, simultaneously. However, some limitations were encountered, e.g., insertion of eight or more hydrophobic aa residues affected both the translocation process across the inner membrane and the assembly process into the outer membrane. Also, the insertion of sequences containing many charged residues resulted in accumulation of precursor protein in the cytoplasm.

INTRODUCTION

Recombinant DNA techniques can be employed for the production of antigens or antigenic determinants from pathogenic organisms in a safe and fast-growing organism such as *E. coli* K-12. We are exploring the possibilities of using PhoE outer-membrane protein of *E. coli* as a carrier for foreign antigenic determinants and permitting their transport to the bacterial cell surface. PhoE protein is an abundant outer-membrane protein of *E. coli* K-12. Its synthesis is induced when cells are grown under phosphate limitation (Overbeeke and Lugtenberg, 1980). The protein forms transmembrane channels through which small hydrophilic solutes can pass (Benz and Bauer, 1988). According to a model for the folding of PhoE protein (Van der Ley et al., 1986; Van der Ley and Tommassen, 1987; Tommassen, 1988), the polypeptide traverses the outer membrane 16 times in antiparallel β-sheet structure, thereby exposing eight regions at the cell surface. If these exposed regions could be exploited for insertion and expression of antigenic determinants at the cell surface this would open possibilities for the development of new types of (live oral) vaccines or serodiagnostic reagents. For this goal several conditions have to be fulfilled, i.e., the biogenesis of the mutant proteins should not be disturbed by the insertions

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); Cm, chloramphenicol; ELISA, enzyme-linked immunosorbent assay; FMDV, foot-and-mouth-disease virus; kb, kilobase(s) or 1000 bp; L-broth, Luria broth; mAb, monoclonal antibody; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; PhoE, phosphate-limitation-inducible outer-membrane pore protein; SDS, sodium dodecyl sulfate; VP, viral protein; wt, wild type.

0378-1119/90/$03.50

and the inserted determinants have to be antigenic and immunogenic in this PhoE-associated conformation.

Previously, we have inserted different combinations of two antigenic determinants of VP1 protein of FMDV, into the fourth and the fifth exposed regions of PhoE protein (Agterberg et al., 1987a; 1990). The hybrid proteins were correctly incorporated into the outer membrane, although the insertions had some adverse effect on the viability of the cells. The inserted epitopes were exposed at the cell surface and recognized by two virus-specific mAbs from the outside of the cell. Immunization experiments in mice, using partially purified proteins, showed that the FMDV determinants in all constructs were immunogenic.

The aim of the present study was to explore the various possibilities for use of PhoE protein as an exposure vector, by inserting antigenic determinants in different exposed regions and making hybrid constructs, containing insertions at two different sites of the protein simultaneously. In addition, we evaluated the maximal length of the inserted epitopes and the effect of the nature of the inserted aa residues.

MATERIALS AND METHODS

(a) Bacterial strains, growth conditions and phages

E. coli K-12 strains CE1224 (Tommassen et al., 1983) and CE1248 (Korteland et al., 1985) produce no pore proteins due to mutations in the *phoE* and *ompR* genes. Strain CE1248 contains in addition a *phoR* mutation resulting in constitutive expression of the *pho* regulon. Cells were grown overnight at 30°C or 37°C under aeration in L-broth (Tommassen et al., 1983) or in a phosphate-limited medium described by Levinthal et al. (1962) to induce the synthesis of PhoE protein. Where necessary, the medium was supplemented with Cm (25 μg/ml) or Ap (50 μg/ml). Adsorption of the PhoE-specific phage TC45 (Chai and Foulds, 1978) to cells was determined as described (Van der Ley et al., 1985).

(b) Construction of the plasmids

The DNA techniques used are as described previously (Agterberg et al., 1989). The plasmids pJP29-M1 (Bosch and Tommassen, 1987) and pJP322 (Bosch et al., 1989a) contain insertions of 12-mer *Bam*HI linkers in the DNA encoding the second and eighth exposed regions of PhoE protein, respectively. Both plasmids were digested with *Bam*HI and dephosphorylated. Two complementary oligos encoding aa 144–150 of VP1 protein of FMDV (see Fig. 1), were phosphorylated and ligated into the linearized plasmids. The linker was designed such that a new *Bam*HI restriction site is generated at the 3′ end of the linker but not at the 5′ end (see Fig. 1). Thus, by repeating the procedure, plasmids could be constructed containing multiple insertions of the linker (see Table I).

```
A
                              73       *
second exposed region:  K T R G S A R
                             314       *
eighth exposed region:  L N P G S G I

B
            144                                   150
   G    S    G    D    L    G    S    L    A    G    S
  GGA  TCT  GGT  GAT  TTA  GGA  TCT  TTA  GCT  GGA  TCC
  CCT  AGA  CCT  CTA  AAT  CCT  AGA  AAT  CGA  CCT  AGG
                                                 BamHI
```

Fig. 1. Sequences of the relevant parts of the second and the eighth exposed regions of PhoE protein and the sequence of the FMDV epitope. (Part A) The aa of PhoE around the site of insertion are indicated, the aa of the 12-mer *Bam*HI linkers are indicated in bold-face letters. The numbers correspond to the positions in the mature protein and the asterisks indicate the site of insertion. (Part B) The aa 144 to 150 of the epitope of VP1 protein of FMDV and the two complementary 27-mer oligos encoding these aa are indicated. The boxed nt are from the vectors. The position of the *Bam*HI site is indicated.

Plasmid pMR05 (Agterberg et al., 1987b) contains a unique *Nru*I restriction site in the DNA encoding the fourth exposed region of PhoE protein. The plasmid was linearized with *Nru*I, dephosphorylated and ligated to the phosphorylated linkers shown in Fig. 2. The 'hydrophobic' linker is self-complementary and encodes 4 hydrophobic aa. The 'hydrophilic' linker also encodes 4 aa, the nature of which depends upon the orientation of insertion. This linker contains an *Eco*RI site. By partial *Eco*RI digestion of plasmids containing multiple insertions and re-ligation, plasmids with smaller insertions could be obtained. The important features of the mutants are summarized in Table II.

Plasmid pMR11 (Agterberg et al., 1987a) contains a linker insertion, encoding an antigenic determinant of VP1 protein of FMDV, in the DNA encoding the fourth cell-surface-exposed region of PhoE protein. This determinant is recognized by mAb MA18. Plasmids pM1-2 and pS2 (see Table II) contain linker insertions in the DNA encoding the second and eighth exposed regions of PhoE protein, respec-

```
'hydrophobic' linker
                 L   A   L   V
              A CTA GCG CTA GT
              T GAT CGC GAT CA

'hydrophilic' linker
              orientation 1          orientation 2

                 R   N   S   R          V   N   S   D
              T CGG AAT TCA CG       C GTG AAT TCC GA
              A GCC TTA AGT GC       G CAC TTA AGG CT
                   EcoRI                  EcoRI
```

Fig. 2. The sequences of the 'hydrophobic' and the 'hydrophilic' linkers. The linkers are designed such that insertion in the *Nru*I site of the *phoE* gene is in the correct reading frame. The hydrophobic linker is self-complementary. The hydrophilic linker codes for different aa in the two possible orientations. The position of the *Eco*RI site is indicated.

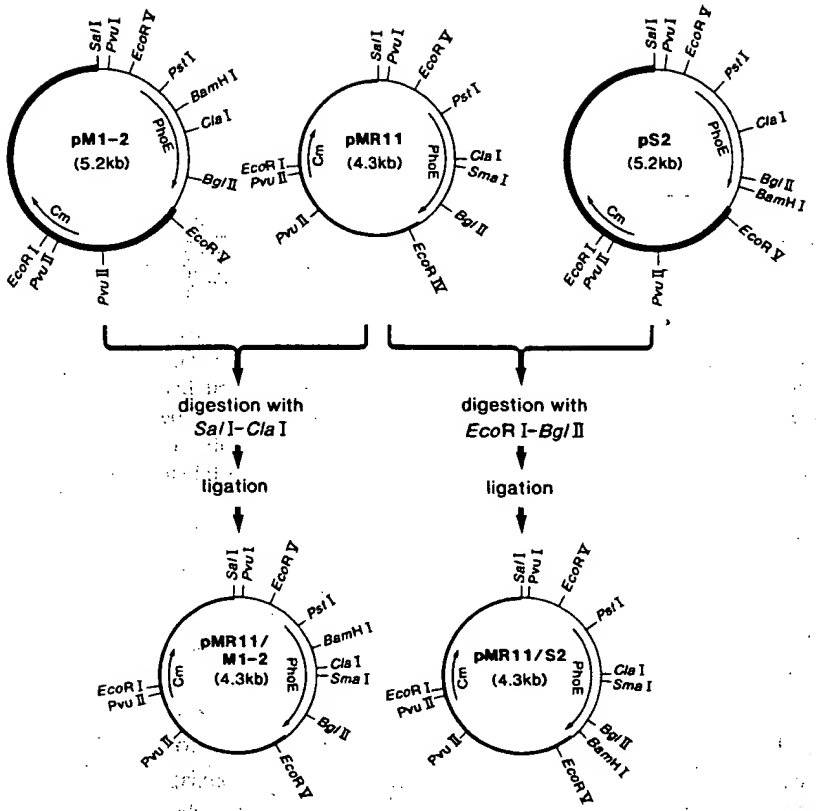

Fig. 3. Construction of plasmids containing insertions at two different sites simultaneously. pMR11 and pM1-2 were digested with *Sal*I + *Cla*I and pMR11 and pS2 were digested with *Eco*RI + *Bgl*II. The samples were loaded on a 1% low-melting-point agarose gel. In both cases the large fragments of pMR11 and the small fragments of pM1-2 and pS2 were sliced out of the gel, the fragments were ligated and used to transform strain CE1224 selecting for Cm-resistant colonies.

tively. Double-mutant plasmids were constructed as depicted in Fig. 3. The resulting plasmids were designated pMR11/M1-2 and pMR11/S2.

(c) Characterization of the hybrid proteins

Protein patterns were analyzed by SDS–PAGE (Lugtenberg et al., 1975) and/or Western immunoblotting (Agterberg et al., 1988). The ultimate localization of the hybrid proteins was determined by whole-cell ELISAs (Van der Ley et al., 1985) and trypsin accessibility experiments (Agterberg et al., 1989b). Pulse-labelling experiments and immunoprecipitation of [^{35}S]methionine-labelled proteins using a polyclonal anti-PhoE serum were performed as described (Bosch et al., 1989b).

(d) In vitro translocation and processing

In vitro transcription, translation and translocation reactions were performed as described (De Vrije et al., 1987). Inverted vesicles of the cytoplasmic membrane of strain MRE600 were added cotranslationally and import of the proteins into the vesicles was demonstrated by their protection against externally added proteinase K. PhoE proteins and PhoE degradation products were subsequently immunoprecipitated and analyzed by SDS–PAGE followed by autoradiography. The relative amounts of the proteins were determined by scanning the bands on the original autoradiogram with a densitometer (Utroscan XL, LKB).

RESULTS AND DISCUSSION

(a) Multiple insertions in the second and eighth exposed region of PhoE protein

Previously, we described the insertion of foreign antigenic determinants in the fourth and in the fifth cell-surface-exposed regions of PhoE protein (Agterberg et al., 1987a; 1990). Even the largest insertions, which consisted of 32 and 14 aa, did not interfere with the assembly of PhoE into the outer membrane. We now wanted to assess whether other exposed regions of PhoE are also suitable for the expression of antigenic determinants at the cell surface and whether there is an upper limit with respect to the number of aa that can be inserted.

Two series of recombinant plasmids were constructed, containing multiple insertions of a 27-mer linker in the DNA coding for the second and the eighth exposed region of PhoE protein, respectively (Table I). This linker encodes

TABLE I

Characteristics of CE1224 cells, expressing hybrid PhoE proteins containing multiple insertions of an antigenic determinant of FMDV

Plasmid[a]	Number of inserted aa	Production[b]	ELISA[c]		Phage adsorption[d]	Trypsin accessibility[e]
			PP1-3	MA11		
pJP29-M1	4	+ +	+ +	−	−	−
pM1-1	13	+ +	+ +	−	−	±
pM1-2	22	+ +	+ +	+	+	±
pM1-3	31	+ +	+ +	+ +	+	±
pM1-4	40	+	+ +	+ +	+	±
pM1-5	49	+	−	+	−	±
pM1-6	58	+	−	+	−	+
pJP322	4	+ +	+ +	−	+	−
pS1	13	+ +	+ +	+ +	+	−
pS2	22	+ +	+ +	+ +	+	−
pS3	31	+	−	+	−	±
pS4	40	+	−	+	−	+
pS5	49	+	−	+	−	+
pS6	58	+	−	+	−	+

[a] Plasmids are described in MATERIALS AND METHODS, section b.

[b] Cells were grown at 30°C in phosphate-limited medium. Cell envelopes were isolated and analyzed by 0.1% SDS–PAGE and Western immunoblotting. Production of the proteins, detected on stained gels, is indicated as + + and + for normal and low production of the proteins, respectively.

[c] The binding of the mAbs was tested in ELISAs, using whole cells as the immobilized antigens. + +, good binding; +, intermediate binding; −, no binding.

[d] Adsorption of phage TC45 to cells. +, adsorption; −, no adsorption.

[e] Trypsin accessibility experiments were performed on cell envelopes. −, no degradation; ±, partial degradation; +, complete degradation.

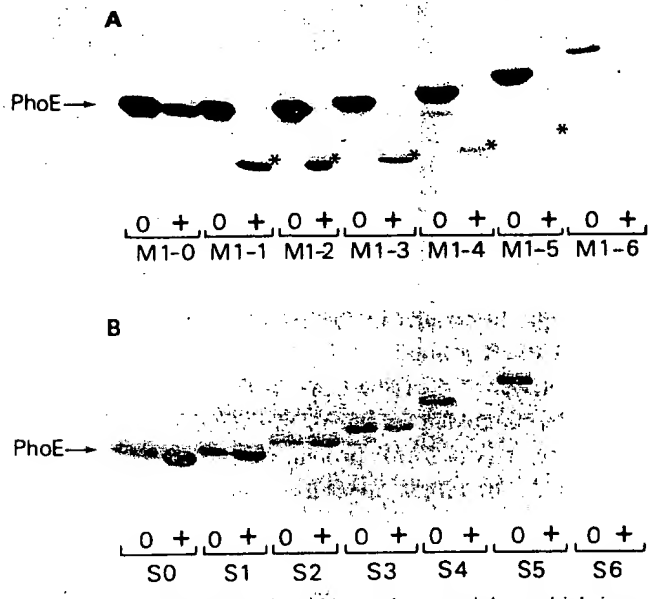

Fig. 4. Trypsin accessibility of hybrid proteins containing multiple insertions of an epitope. Cell-envelope preparations of cells containing different plasmids were treated with trypsin and analyzed by 0.1% SDS–PAGE followed by Western immunoblot using a polyclonal anti-PhoE serum. (Panel A) Cells expressing the hybrid proteins encoded by pJP29-M1 to pM1-6. (Panel B) Cells expressing the hybrid proteins encoded by pJP322 to pS6. 0, no trypsin treatment; +, trypsin treatment. The positions of the PhoE degradation products are indicated by asterisks. Only the relevant parts of the blots are shown.

an antigenic determinant of VP1 protein of FMDV (Fig. 1), which reacts with the virus-specific mAb MA11 (Meloen et al., 1987). The plasmids were designated pM1-n and pSn for insertions in the DNA encoding the second and eighth exposed regions, respectively, where n represents the number of linkers inserted.

The level of production of the different hybrid proteins was tested in strain CE1224 (Table I, Fig. 4). The hybrid proteins co-fractionated with the cell envelopes. Production of the larger hybrid proteins, M1-5, M1-6 and S3 to S6 was rather weak, but was markedly increased when the strains were grown at 30°C instead of 37°C. Transformation of *phoR* strain CE1248 with the different recombinant plasmids yielded colonies which were markedly smaller in size than colonies of CE1248 transformed with the vector plasmids. This suggests that production of these hybrid proteins is somewhat harmful to the cells. In addition, repeated analysis of the cell-envelope patterns of the transformants revealed that there is a selection against the production of the larger hybrid proteins in this constitutive strain resulting in the deletion of variable parts of the insert.

In whole-cell ELISAs, cells expressing M1-0 to M1-4 and S0 to S2 were recognized by the PhoE-specific mAb PP1-3 (Table I). Since this mAb recognizes a conformational epitope in the cell-surface-exposed part of PhoE

(Van der Ley et al., 1985; 1986), it appears that these hybrid proteins are correctly assembled into the outer membrane. Cells expressing the larger hybrid proteins did not bind PP1-3. The reaction with MA11 increased with the number of insertions, with a maximal reaction when the linker was inserted four times in the second exposed region or twice in the eighth exposed region. When the weak production of the M1-5, M1-6 and S3 to S6 proteins is taken into account, a rather good reaction with MA11 was still observed, suggesting that also these hybrid proteins are incorporated into the outer membrane. However, since production of these hybrid proteins apparently affected the viability of the cells, we cannot exclude that some cell lysis had occurred, thereby exposing periplasmically located proteins. Also, the binding of the PhoE-specific phage TC45 was tested (Table I). Previously, it has been shown that insertion of extra aa in the second exposed region of PhoE blocks the phage receptor function (Bosch and Tommassen, 1987). Consistently, cells expressing the proteins M1-0 and M1-1 did not bind the phages, but cells expressing the proteins M1-2 to M1-4 did. Apparently, the receptor function of these proteins has been restored due to the extra aa inserted. Cells expressing the proteins S0 to S2 did also bind the phage. These results confirm that the proteins M1-2 to M1-4 and S0 to S2 are correctly assembled into the outer membrane. Cells expressing the larger proteins, M1-5, M1-6 and S3 to S6 did not bind the phages, indicating that these proteins are either not correctly incorporated into the outer membrane or that the phage receptor function is disturbed by the insertions.

Trypsin accessibility experiments were further employed to determine whether the hybrid proteins are assembled into the outer membrane. When wt PhoE protein is correctly incorporated into the outer membrane it is protected against trypsin treatment of cell envelopes (Tommassen and Lugtenberg, 1984). Mutant proteins which are not correctly assembled are completely degraded (Agterberg et al., 1989). Insertion mutations may create exposed trypsin-sensitive sites. When a mutant protein containing such an insertion is correctly assembled into the outer membrane, one may expect to find protected fragments of the protein after treatment of the cell envelopes with trypsin (Morona et al., 1985; Freudl et al., 1986). Hybrid proteins M1-0 to M1-5 and S0 to S3 were at least partially protected against trypsin treatment (Fig. 4). In case of the hybrid proteins M1-1 to M1-5 stable degradation products were detected, approx. 8 kDa smaller in size than the intact proteins. Since the linkers in this series of proteins are inserted between aa residues 74 and 75 of PhoE, the protected degradation products correspond to the C-terminal fragment of PhoE, which is cleaved at the site of insertion. It should be noted that in case of hybrid protein M1-5 only a very weak band could be detected, indicating that only a minor part of the protein is incorporated into the outer membrane. The hybrid proteins M1-6 and S4 to S6 were completely degraded, indicating that these proteins are not incorporated into the outer membrane.

In conclusion, antigenic determinants can be inserted in the second and eighth cell-surface-exposed regions of PhoE protein. The mutant proteins are correctly assembled into the outer membrane and the inserted determinants are exposed. The maximal number of aa that can be inserted without disturbing outer membrane assembly is dependent on the site of insertion. In case of the second exposed region approx. 50 aa can be inserted. This number corresponds fairly well to the maximal number of aa that can be inserted in a cell surface-exposed region of another outer-membrane protein, LamB (Charbit et al., 1988). However, in the eighth exposed region of PhoE, substantially less aa (about 30) can be inserted. It is well possible that the nature of inserted aa can affect the maximal length of the insert.

(b) Insertion of hydrophobic aa residues

PhoE protein, like other outer-membrane proteins, contains no stretches of hydrophobic aa residues long enough to span the membrane as an α-helix. Its membrane-spanning segments are mostly amphipathic β-sheets. It has been shown that stretches of 16–18 hydrophobic aa can block the translocation of outer-membrane protein OmpA across the inner membrane, by acting as an anchor sequence (MacIntyre et al., 1988). To investigate whether hydrophobic insertions can interfere with PhoE biogenesis, a linker encoding 4 hydrophobic aa (Fig. 2), was inserted in the fourth exposed region of PhoE protein. Three plasmids, pMR5H1 to pMR5H3, containing insertions of one, two or three times the linker, were obtained.

The level of production of these hybrid proteins in strain CE1224, after growth of the cells under phosphate limitation, was comparable to the wt PhoE protein level. When *phoR* strain CE1248 was transformed with the different plasmids, only very small colonies were obtained. Moreover, the level of production of the proteins H2 and H3 was very much decreased. In whole-cell ELISAs using plasmid-containing CE1224 cells, binding of the PhoE-specific mAb PP1-3 was observed, but the reaction with cells expressing proteins H2 and H3 was strongly diminished. Consistent with these results, protein H1 was completely protected against trypsin treatment of cell envelopes. However, proteins H2 and H3 were, respectively, partially or completely degraded and no protected products were detected (Fig. 5). Trypsin accessibility experiments on whole cells in the presence of EDTA confirmed these data. EDTA permeabilizes the outer membrane and, consequently, trypsin has access to the periplasm. In these experiments no protected C-terminal fragment could be detected. These results show that the assembly into the outer membrane of protein H1, which contains 4 extra aa residues, is normal, but that the

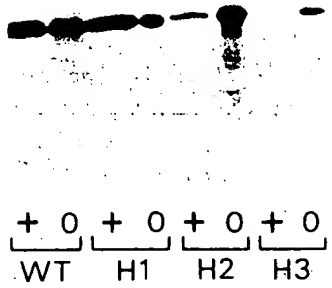

Fig. 5. Trypsin accessibility experiments of hybrid proteins, containing hydrophobic insertions. Cell-envelope preparations of CE1224 cells expressing the hybrid proteins encoded by pMR05 (WT), pMR5H1 (H1), pMR5H2 (H2) and pMR5H3 (H3) were treated (+) or not treated (0) with trypsin. The samples were analyzed as in Fig. 4. Only the relevant part of the blot is shown.

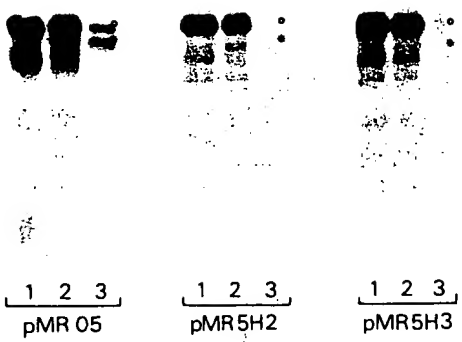

Fig. 6. In vitro transcription, translation and translocation of PhoE derivatives. Lanes: 1, translation products of plasmids pMR05, pMR5H2 and pMR5H3 after incubation in the cell free-system; 2, translation products after incubation in the presence of cotranslationally added inner-membrane vesicles; 3, translation products protected against subsequent addition of proteinase K. The samples were analyzed by SDS–PAGE after immunoprecipitation. The positions of precursor PhoE and mature PhoE are marked with circles and asterisks, respectively. Only the relevant part of the autoradiogram is shown.

assembly of the proteins H2 and H3, which contain 8 and 12 extra aa residues, respectively, is strongly disturbed.

To determine whether translocation across the inner membrane is also disturbed, in vitro transcription, translation and translocation experiments using inner-membrane vesicles were performed. When the inserted lipophilic stretches are serving as membrane anchors with respect to the inner membrane the translocation efficiency might be disturbed. pMR05, which encodes wt PhoE protein, was used in a cell-free system to direct protein synthesis and a PhoE-specific band, representing the precursor could be detected (Fig. 6). Approximately 70% of the precursor was imported into cotranslationally added vesicles of the cytoplasmic membrane, as was demonstrated by their protection against proteinase K treatment. In case of the mutant proteins H2 and H3 only approx. 20% of the synthesized proteins was translocated (Fig. 6) and no protected N-terminal fragment could be detected. Also in vivo pulse-chase experiments were performed. When cells expressing wt PhoE protein were pulse-labelled with [^{35}S]methionine during 30 s, about 10% of the total amount of PhoE was in the precursor form and 90% in the mature form. The precursor completely disappeared during a 2-min chase period. In pulse-label experiments, accumulation of precursor proteins H2 and H3 was observed (results not shown), however the effect in vivo was not as drastic as in vitro. This may be due to the fact that cells can adapt to

TABLE II

Composition of the insertions of the 'hydrophilic' linker in the fourth exposed region of PhoE protein

Plasmid[a]	Number and sequence of inserted aa[b]		ELISA[c] PP1-3	Trypsin accessibility[d]
pMR5C1	4	VNSD	+	–
pMR5C2	8	RNSRRNSR	+	*
pMR5C3	8	RNSDRNSR	+	–
pMR5C4	12	RNSRVNSDRNSR	+	*
pMR5C5	12	VNSDVNSDVNSD	+	–
pMR5C6	16	VNSDVNSDVNSDVNSD	+	–
pMR5C7	16	RNSDRNSRRNSRRNSR	+	*
pMR5C8	20	RNSRRNSRRNSRRNSRRNSR	–	*
pMR5C9	28	RNSRRNSRVNSDVNSDRNSRRNSRRNSR	+	*
pMR5C10	28	VNSDVNSDRNSRVNSDVNSDVNSDVNSD	–	ND

[a] Plasmids are described in MATERIALS AND METHODS, section b.

[b] The number of aa inserted and the sequences of the insertions are given. The linker is described in MATERIALS AND METHODS, section b.

[c] The binding of mAb PP1-3 was tested in whole-cell ELISAs. +, binding; –, no binding.

[d] Trypsin accessibility experiments were performed on whole cells and cell-envelope preparations. –, no degradation; *, degradation resulting in protected products; ND, not done.

stress situations caused by accumulating precursors by the overproduction of several proteins involved in the transport process (Moreno et al., 1980). Although stretches of eight to twelve hydrophobic aa do affect the transport efficiency out of the cytoplasm they seem to be too short to function as a real stop-transfer sequence, since no protected N- or C-terminal fragments were found in in vitro and in vivo protease accessibility experiments, respectively. Thus, the insertion of stretches of eight or more hydrophobic aa residues in the fourth exposed region of PhoE protein interferes both with translocation across the inner membrane and with assembly into the outer membrane. It should be noted that antigenic determinants are in general rather hydrophilic (Berzofsky, 1985). Therefore, we do not consider this result as a severe limitation to the use of PhoE protein as an expression vector.

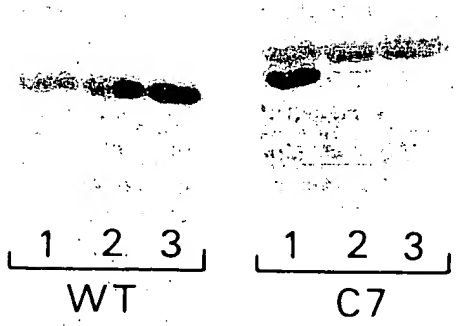

Fig. 8. Trypsin accessibility experiments on whole cells. Cells expressing wt PhoE and C7 were either not treated with trypsin (lanes 1) or treated with trypsin in the presence of $MgCl_2$ (lanes 2) or EDTA (lanes 3). Protein patterns were analyzed by SDS–PAGE followed by Western immunoblot, using a polyclonal anti-PhoE serum. Only the relevant part of the blot is shown.

(c) **Insertion of hydrophilic, charged aa residues**

To investigate the influence of hydrophilic, charged aa residues on the biogenesis of PhoE, pMR5C1 to pMR5C10 (Table II) were constructed. The mutant proteins encoded by pMR5C1 to pMR5C7 and pMR5C9 were normally assembled into the outer membrane: intact cells expressing the corresponding proteins bound the PhoE-specific mAb PP1-3 and, after trypsin treatment of cell envelopes, either the whole protein or two fragments were protected against digestion (as shown for C4, C7 and C9 in Fig. 7). In most cases a precursor form of the mutant protein was visible, in addition to the mature form, when cell-envelope proteins were analyzed by SDS–PAGE. This indicates that translocation across the inner membrane and/or digestion by leader peptidase I is retarded. To localize the accumulated precursors, whole cells were incubated with trypsin in the presence of EDTA. Under these conditions, the accumulated precursors were not degraded (as was shown for C7 in Fig. 8). Therefore, the insertions interfere with the efficiency of export out of the cytoplasm.

Two mutant proteins, i.e., those encoded by pMR5C8 and pMR5C10, behaved differently. Cells expressing the pMR5C8-encoded protein did not bind the PhoE-specific mAb PP1-3 in ELISAs. Upon tryptic digestion of cell-envelope preparations the protein was degraded and only one protected fragment was observed (Fig. 7, C8). In Western immunoblots, this fragment reacted with mAb mE1, for which the epitope is located between aa 47 and 55. Apparently, the N-terminal segment of the mutant protein is normally incorporated into the outer membrane and protected against trypsin digestion, whereas the insertion interferes with the assembly of the C-terminal part of the protein. Comparison of the proteins encoded by pMR5C8 and pMR5C9 reveals an interesting feature. The former protein contains an insertion of 20 aa including 10 positively charged aa residues. In the latter protein this insertion is interrupted by an additional insertion of 8 aa including two negatively charged residues (Table II). In contrast to C9 hybrid protein C8 is not correctly incorporated into the outer membrane. Outer-membrane proteins are in general negatively charged. For instance, the net charges of the related outer-membrane proteins OmpC, OmpF and PhoE are −14, −11 and −9, respectively (Mizuno et al., 1983). Due to the insertion, protein C8 has a net positive charge of + 1, whereas the larger mutant protein C9 is still negatively charged (−1). These results suggest that the net negative charge of the outer membrane proteins is important for outer-membrane localization.

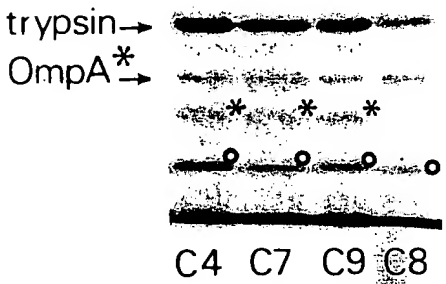

Fig. 7. Trypsin accessibility experiment. Cell-envelope preparations of cells containing plasmids pMR5C4, pMR5C7, pMR5C8 and pMR5C9 were treated with trypsin and analyzed by SDS–PAGE. The positions of trypsin and the protected OmpA fragment (OmpA*) are indicated. The protected N- and C-terminal fragments of PhoE are marked with circles and asterisks, respectively. Only the relevant part of the gel is shown.

No PhoE band could be detected when cell-envelope preparations of cells expressing pMR5C10 were analyzed by SDS–PAGE and, consistently, the PhoE-specific mAb PP1-3 did not bind to these cells. However, in pulse-label experiments a truncated PhoE product with an estimated size of 17 kDa and an apparent precursor form were visible (Fig. 9, C10). The precursor disappeared during a 2-min chase period, indicating transport across the inner membrane. During longer chase periods, the mature form also disappeared, apparently due to proteolytic degradation. In cells expressing plasmids pMR5C5 and pMR5C6, which were generated from pMR5C10 by partial digestion with *Eco*RI, a normal sized PhoE product was present (Fig. 9, C5 and C6). Moreover, in cells expressing pMR5C5 also a truncated PhoE product could be detected. Apparently, for unknown reasons, a complete or partial transcription or translation stop occurs within the insertions in pMR5C10 and pMR5C5.

(d) Insertions at two different sites of PhoE protein

To investigate whether it is possible to make insertions in two exposed regions of PhoE simultaneously, two double-mutant plasmids, pMR11/M1-2 and pMR11/S2, were constructed (Fig. 3). In pulse-chase experiments the pMR11/M1-2-encoded protein appeared to be very instable; during a pulse period of 30 s both a precursor PhoE and a truncated PhoE product of approx. 17 kDa could be detected, but after a chase period of 10 min both PhoE products were completely degraded. On the other hand, the pMR11/S2-encoded protein appeared to be stable. In whole-cell ELISAs, cells expressing this protein were recognized by the PhoE-specific mAb PP1-3 and by the virus-specific mAbs MA11 and MA18. Trypsin accessibility experiments performed on cell-envelope preparations showed that part of the protein was protected against digestion and that part of the protein was degraded, resulting in two protected degradation products. Apparently, this protein is correctly assembled into the outer membrane. We expect that the simultaneous production of different antigenic determinants and multiple copies of these determinants, will reduce the chance on selection of 'escape'-mutants and enhance the immune response when these constructs are used in vaccine preparations.

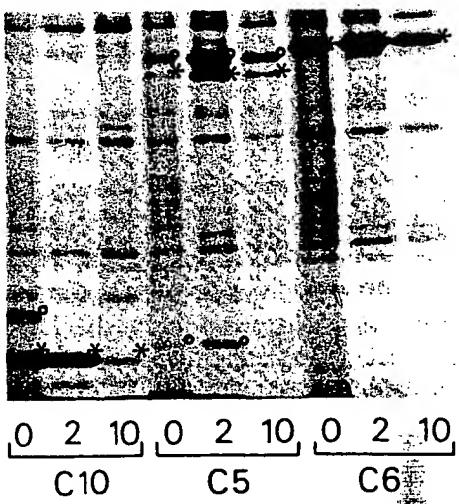

Fig. 9. Autoradiogram of radioactively labelled cellular proteins separated by SDS–PAGE. Cells containing pMR5C5, pMR5C6 and pMR5C10 were pulse-labelled with [^{35}S]methionine for 30 s, followed by chase periods of 2 and 10 min as indicated. The positions of PhoE precursor and mature PhoE protein are marked with circles and asterisks, respectively.

(e) Conclusions

Here, we have assessed the possibilities and limitations of using PhoE protein as a carrier for the expression of foreign antigenic determinants and their transport to the cell surface. Although some insertions interfered with the assembly of PhoE, it is evident that the PhoE expression system is very flexible. Four exposed regions were found to be suitable for insertion of extra aa and two sites can be used simultaneously. Previously, we have shown that a partially purified hybrid protein, containing an insertion of two antigenic determinants of VP1 protein of FMDV, elicited high levels of neutralizing antibodies in guinea pigs and protected the animals against challenge with virus (manuscript submitted for publication). Moreover, we have shown that *E. coli* PhoE is normally produced and incorporated into the outer membrane of two attenuated *Salmonella typhimurium* strains (Agterberg et al., 1988). Thus, the possibility of inserting one or more foreign antigenic determinants in cell-surface-exposed regions of PhoE and the subsequent presentation at the bacterial cell surface can open the way for the development of new types of live oral vaccines.

ACKNOWLEDGEMENTS

This work was partially supported by the Foundation for Medical and Health Research (MEDIGON), grant No. 900-515-003.

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